

Application  
for  
United States Letters Patent

098099920-031601

To all whom it may concern:

Be it known that Taka-Aki Sato

have invented certain new and useful improvements in

TREX, A NOVEL GENE OF TRAF-INTERACTING EXT GENE FAMILY AND DIAGNOSTIC AND THERAPEUTIC  
USES THEREOF

of which the following is a full, clear and exact description.

TREX, A NOVEL GENE OF TRAF-INTERACTING EXT GENE FAMILY AND  
DIAGNOSTIC AND THERAPEUTIC USES THEREOF

This application claims priority and is a continuation-in-part application of U.S. Serial No. 09/156,191, filed September 17, 1998, the contents of which is hereby incorporated by reference.

## STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

The invention disclosed herein was made in part with Government support under NIH Grant No. R01GM55147. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

## BACKGROUND OF THE INVENTION

Tumor necrosis factor (TNF) receptor-associated factor (TRAF) proteins contribute to signal transduction induced by TNF receptor family signaling. TRAF3 cloned as binding protein to the cytoplasmic domain of CD40, a member of TNF receptor superfamily, is believed to be involved in signaling pathway induced by CD40, Lymphotoxin (LT)  $\beta$  receptor, CD30 ligation (1-7). Here we report molecular cloning of a novel TRAF-interacting protein named as TREX because of TRAF-interacting EXT (hereditary multiple exostoses) gene family protein. TREX has highly homologous sequence to the EXT gene family, a candidate of tumor

suppressor gene. TREX strongly interacts with TRAF2 and TRAF3, and TREX and TRAF protein colocalize in mammalian cells. Moreover, overexpression of TREX modulates NF-kB activity induced by TRAF-mediated signaling. These findings indicate that TREX and the other EXT gene family proteins can function as a mediator in receptor signaling and could be involved in tumorigenesis.

#### SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

This invention provides an isolated nucleic acid molecule encoding a mutant homolog of the mammalian Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein whose mutant sequences (genetic alterations) are shown in Table 3 infra.

This invention provides a vector comprising the isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

This invention provides a purified mammalian Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

This invention provides a protein comprising substantially the amino acid sequence set forth in Figure 1A (SEQ ID NOS:2 and 4).

This invention provides an oligonucleotide comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of an isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

This invention provides an antisense oligonucleotide comprising a sequence capable of specifically hybridizing with a unique sequence included within an mRNA molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

This invention provides an antisense oligonucleotide comprising a sequence capable of specifically hybridizing with a unique sequence included within a genomic DNA molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

This invention provides a monoclonal antibody directed to an epitope of a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

This invention provides a method of inhibiting TREX protein interaction with a TRAF protein comprising administering a ligand comprising an amino acid domain which binds to a EXT C domain of the TREX protein so as to inhibit binding of the TREX protein to the TRAF protein.

This invention provides a method of inhibiting overexpression of TREX protein comprising administering any of the above-described antisense oligonucleotides which bind to an mRNA molecule encoding a human Tumor necrosis factor

Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TRES) protein so as to inhibit overexpression of the human TRES protein.

5 This invention provides a method of inhibiting growth of a tumor cell comprising blocking a TRAF interacting site of a TRES protein by administering a ligand capable of binding to the TRAF interacting site of a TRES protein.

10 This invention provides a pharmaceutical composition comprising an amount of any of the above-described oligonucleotides effective to prevent overexpression of a TRES protein and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

15 This invention provides a pharmaceutical composition comprising an amount of any of the above-described antibodies effective to block binding of a TRES protein to a TRAF protein and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

20 This invention provides a method of treating an abnormality in a subject, wherein the abnormality is alleviated by the inhibition of binding of a TRES protein and a TRAF protein which comprises administering to the subject an effective amount of the above described pharmaceutical composition effective to block binding of the TRES protein and the TRAF protein in the subject, thereby treating the abnormality in the subject.

30 This invention provides a method of treating an abnormality in a subject, wherein the abnormality is alleviated by the inhibition of overexpression of a TRES protein which comprises administering to the subject an effective amount of the above-described pharmaceutical composition effective to inhibit overexpression of the TRES protein, thereby treating the abnormality in the subject. In a preferred

embodiment the abnormality is cancer, a hereditary multiple extosis or an autoimmune disease.

5 This invention provides a method of screening for a chemical compound which inhibits TREX protein and TRAF protein binding comprising: (a) incubating the chemical compound with a TREX protein and a TRAF protein; (b) contacting the incubate of step (a) with an affinity medium under conditions so as to bind a TREX protein-TRAF protein complex, if such a complex forms; and (c) measuring the amount of the TREX protein-TRAF protein complex formed in step (b) so as to determine whether the compound is capable of interfering with the formation of the complex between the TREX protein-TRAF protein.

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15 This invention provides a method of preventing inhibition of a CD40 signal-dependent NF-kB activation comprising administering any of the above-described antisense oligonucleotides which bind to an mRNA molecule encoding a human Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein so as to prevent inhibition of CD40 signal-dependent NF-kB activation.

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25 This invention provides a method of preventing inhibition of activation of a CD40 signal-dependent NF-kB comprising administering a ligand comprising an amino acid domain which binds to a EXT C domain of the TREX protein so as to inhibit binding of the TREX protein to the TRAF protein, thereby preventing inhibition of activation of a CD40 signal-dependent NF-kB.

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35 This invention provides a method of preventing upregulation of a TNF receptor typeII signal-dependent NF-kB activation comprising administering any of the above-described antisense oligonucleotides which bind to an mRNA molecule encoding a human Tumor necrosis factor Receptor-Associated

Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein so as to prevent upregulation of a TNF receptor typeII (TNFRII) signal-dependent NF-kB activation.

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This invention provides a method of preventing upregulation of activation of a TNF receptor typeII (TNFRII)-signal-dependent NF-kB comprising administering a ligand comprising an amino acid domain which binds to a EXT C domain of the TREX protein so as to inhibit binding of the TREX protein to the TRAF protein, thereby preventing upregulation of activation of a TNF receptor typeII-signal-dependent NF-kB.

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This invention provides a method of detecting a predisposition to cancer which comprises detecting of a mutation in a nucleic acid encoding TREX protein in the sample from the subject.

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This invention provides a TREX nucleic acid probe comprising a sequence capable of specifically hybridizing with a unique sequence included within the above-described isolated DNA molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

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This invention provides a method of diagnosing cancer in a subject which comprises: a) obtaining DNA from the sample of a subject suffering from cancer; b) performing a restriction digest of the DNA with a panel of restriction enzymes; c) separating the resulting DNA fragments by size fractionation; d) contacting the resulting DNA fragments with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a genetic alteration of a nucleic acid molecule encoding a TREX protein, wherein the nucleic acid is labeled with a detectable marker; e) detecting labeled bands which have hybridized to the nucleic acid probe in step (d),

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wherein the sequence of a genetic alteration of a nucleic acid molecule encoding a TREX protein creates a unique band pattern specific to the DNA of subjects suffering from cancer; f) preparing DNA obtained from a sample of a subject for diagnosis by steps (a-e); and g) comparing the detected band pattern specific to the DNA obtained from a sample of subjects suffering from cancer from step (e) and the DNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to cancer if the patterns are the same.

This invention provides a method of diagnosing cancer in a subject which comprises: a) obtaining RNA from the sample of the subject suffering from cancer; b) separating the RNA sample by size fractionation; c) contacting the resulting RNA species with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mutated TREX protein, wherein the sequence of the nucleic acid molecule encoding the mutated TREX protein is labeled with a detectable marker; d) detecting labeled bands which have hybridized to the RNA species to create a unique band pattern specific to the RNA of subjects suffering from cancer; e) preparing RNA obtained from a sample of a subject for diagnosis by steps (a-d); and f) comparing the detected band pattern specific to the RNA obtained from a sample of subjects suffering from cancer from step (d) and the RNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to cancer if the patterns are the same.



BRIEF DESCRIPTION OF THE FIGURES

5 **Figures 1A-1F.** Amino acid sequences of TREX and expression  
of TREX. Fig. 1A, Predicted amino acid sequences of mouse  
and human TREX. Identical residues are boxed. Partial clones  
obtained by two-hybrid screening are indicated by brackets.  
Isoleucine and leucine residues that form putative  
10 isoleucine zipper motif are boxed and darkly shaded. Fig.  
1B, Schematic representation of putative domain structure of  
EXT gene family proteins. Conserved domains are indicated as  
EXT-N and EXT-C domain. Fig. 1C, Sequence alignments of  
EXT-N domain. Conserved residues are shaded. Fig. 1D,  
15 Sequence alignments of EXT-C domain. Conserved residues are  
shaded. Fig. 1E, Northern blot analysis of TREX mRNA.  
Multiple tissue northern blot (Clontech) were probed with  
human or mouse TREX cDNA. Fig. 1F, Expression of TREX  
protein in human cells. Cell lysates of KM12L4 cell line  
20 were immunoprecipitated with either rabbit preimmune IgG or  
rabbit anti-TREX antibody. TREX proteins were detected with  
anti-TREX antibody (107 kDa).

**Figure 2A-B.** Intracellular association of TREX and TRAF  
25 family proteins. Fig. 2A, 293 T cells were transiently  
transfected with myc-tagged TREX together with FLAG-tagged  
TRAFs. Cell lysates were immunoprecipitated with preimmune  
rabbit IgG (Control) or rabbit anti-myc antibody ( $\alpha$ myc).  
Coimmunoprecipitated TRAF proteins were analyzed by Western  
30 blotting using anti-FLAG antibody. Expression of TRAF  
proteins was monitored by Western blotting using cell  
lysates (bottom). Fig. 2B, Colocalization of TREX and TRAF3  
in mammalian cells. COS7 cells were transfected with  
myc-tagged TREX or TRAF3. Myc-tagged TREX (R-phycoerythrin,  
35 red) localized around nucleus as similar with TRAF3 (FITC,  
green).

**Figure 3.** TREX modulates NF- $\kappa$ B activity induced by TRAF-mediated signaling pathway. 293 cells were transiently transfected with NF- $\kappa$ B-dependent reporter gene together with several amounts of TREX in the presence of CD40 and CD40 ligand (a) or TRAF2 (b). Luciferase activities were determined and normalized by co-transfection of pRL-CMV using dual-luciferase assay kit (Promega).

**Figure 4.** TREX upregulates NF- $\kappa$ B activity induced by TNF $\alpha$ -induced NF- $\kappa$ B activation in human embryonic kidney 293 cell. 293 human embryo kidney cells were maintained in MEM containing 10% FCS, 100  $\mu$ g/ml penicillin G and 100  $\mu$ g/ml streptomycin. For reporter assay, 10<sup>6</sup> cells were seeded on 100 mm dishes and grown for 3 days in 5% CO<sub>2</sub> at 37° C. The cells were transfected with reporter DNA (luciferase) and either empty (pcDNA3.1(-)/MYC HIS) or mTREX expression plasmid (pcDNA3.1(-)/MYC HIS-m TREX) by the calcium phosphate precipitation method. After 12 h, the cells were treated with or without 20 ng/ml TNF- $\alpha$ . After an additional incubation for 12 h, the cells were washed with PBS and then the luciferase activities were determined by using Dual luciferase reporter assay system (Promega).

**Figure 5.** Chromosomal mapping of the TREX gene on chromosome 8p12-p21. The biotin-labeled TREX cDNA probe and the digoxigenin-labeled chromosome 8 centromere-specific probe were cohybridized to a normal human metaphase (a) or prophase (b) spreads and detected with avidin FITC (green signals) and anti-digoxigenin-rhodamine (red signals), respectively. Chromosomes were counterstained with DAPI (blue).

**Figure 6.** Genomic organization of TREX gene. Exon-intron distribution is shown in upper panel. The 7 exons are indicated by box and numbered. The size of intron is also indicated in kilobases. The middle panel represents the TREX cDNA with translation initiation site (ATG) and termination

site (TAG). Closed box and open box in these represent the coding region and non-coding region, respectively.

**Figures 7A-7B.** Fig. 7A. Mouse TREX cDNA nucleotides 1-3479. (SEQ ID NO: 1); Mouse TREX cDNA Genbank Accession NO. AF083550. Fig. 7B. Mouse TREX cDNA nucleotides and the predicted amino acid sequence (SEQ ID NO: 2).

**Figure 8A-8B.** Fig. 8A. Human TREX cDNA nucleotides 1-6172. (SEQ ID NO: 3); Human TREX cDNA Genbank Accession NO. AF083551. Fig. 8B. Human TREX cDNA nucleotides and the predicted amino acid sequence (SEQ ID NO: 4)

**Figures 9A-9B.** Sequence alignment of mouse and human EXTL3 proteins and expression of mouse EXTL3 and mRNA in various tissues. Fig. 9A. The amino acid sequence of mouse EXTL3 (AF083550) and human EXTL3 (AF083551) were aligned by using GENETYX-MAC 9.0. Identical residues are boxed, and a putative isoleucine zipper motif is shaded. Fig. 9B. Expression of the mouse EXTL3 gene on a commercial Northern blot (Clontech) of eight different tissues using a cDNA fragment as a probe. The various tissues are labeled at the top, and the size markers are indicated on the left. A transcript of about 6kb is present in all tissues.

**Figures 10A-10C.** Enhancement of NF- $\kappa$ B activation stimulated by TNF- $\alpha$  in HEK293 cells overexpressing EXTL3. Fig. 10A. HEK293 cells were transfected with pcDNA or pcDNA/EXTL3. After 12 h, the cells were stimulated with or without 20 ng/ml TNF- $\alpha$  for 1 h. Then, nuclear extracts prepared from the cells were analyzed by using a electrophoretic mobility shift assay with NF- $\kappa$ B consensus oligonucleotide. Fig. 10B. The indicated amount of pcDNA/EXTL3 was cotransfected with 500 ng of the luciferase reporter plasmid pELAM-luc and 500 ng pRL-TK into HEK293 cells. The total amount of pcDNA constructs was adjusted to 10  $\mu$ g by addition of empty vector. After 12 h, the cells were treated with or without

20 ng/ml TNF- $\alpha$ . At 12 h after stimulation, cell lysates were prepared and subjected to a dual luciferase assay. All values representing luciferase activities were normalized and are shown as the mean $\pm$ SEM of triplicate samples. Fig. 10C The indicated amount of pcDNA/EXTL3 and 5  $\mu$ g of HA-tagged human TRAF2 construct were transfected with 500 ng of the luciferase reporter plasmid pELAM-luc and 500 ng pRL-TK into HEK293 cells. The total amount of pcDNA constructs was adjusted to 10 $\mu$ g by adding an empty vector. After 24 h, cell lysates were prepared and subjected to the dual luciferase assay. All values representing luciferase activities were normalized and are shown as the mean $\pm$ SEM of triplicate samples.

**Figures 11A-11Da-11Dc. Effects of EXTL3 truncation mutants on NF- $\kappa$ B activity.** Fig. 11A. Schematic representation of truncation mutants used in this assay. TM, transmembrane region; EXT-C, EXT-COOH domain; EXT-N, EXT-NH<sub>2</sub> domain. Fig. 11B. A 10- $\mu$ g aliquot of pcDNA/EXTL3, pcDNA/ $\Delta$ N EXTL3, pcDNA/ $\Delta$ C EXTL3, or pcDNA/ $\Delta$ N&C EXTL3 was transfected with 500 ng pELAM-luc and 500 ng pRL-TK into HEK293 cells. After 12 h, the cells were treated with (hatched column) or without (open column) 20 ng/ml TNF- $\alpha$ . At 12 h after stimulation, cell lysates were prepared and subjected to the dual luciferase assay. All values representing luciferase activities were normalized and are shown as the mean $\pm$ SEM of six samples. Fig. 11C. A 5 $\mu$ g of pcDNA/EXTL3, pcDNA/ $\Delta$ N EXTL3, pcDNA/ $\Delta$ C EXTL3, or pcDNA/ $\Delta$ N&C EXTL3 and 5  $\mu$ g HA-tagged human TRAF2 construct (hatched column) or empty vector (open column) were transfected with 500 ng pELAM-luc and 500 ng pRL-TK into HEK293 cells. After 24 h, cell lysates were prepared and subjected to the dual luciferase assay. All values representing luciferase activities were normalized and are shown as the mean  $\pm$ SEM of seven samples. Fig. 11D. HEK293 cells cultured on cover glasses were transfected with pEGFP-N2 (a), pEGFP/EXTL3 (b), or pEGFP/ $\Delta$ N EXTL3 (c). After transfection, the cells were fixed with

3.7% formalin. Then, cells were treated with 0.2% Triton X-100. Fluorescence was imaged with a confocal laser scanning microscope. Bar, 50  $\mu$ m.

5     **Figures 12A-12H. Effects of TRAFs on EXTL3 distribution**  
HEK293 cells cultured on cover glasses were transfected with  
EGFP-tagged EXTL3 construct and FLAG-tagged TRAF2 (Figs.  
12A-12D) or TRAF3 (E-H) constructs. After transfection, the  
10     cells were fixed with 3.7% formalin. Then, cells were  
treated with 0.2% Triton X-100. After blocking, indirect  
immuno-fluorescence analysis was performed. Monoclonal  
anti-FLAG antibody was used as a first antibody followed by  
a Cy-5-conjugated second antibody. TRITC-concanavalin A was  
used to reveal the endoplasmic reticulum region.  
15     Fluorescence was imaged with a confocal laser scanning  
microscope. EXTL3 is shown in green (Figs. 12A, 12E). The  
concanavalin A-stained region is shown in red (Figs. 12B,  
12F). Fig. 12C shows TRAF2 in white, and Fig. 12G shows  
TRAF3 in white. Fig. 12D is a merged image of Figs. 12A,  
20     12B, and 12C, and Fig. 12H shows a merged image of Figs.  
12E, 12F, and 12G. Bar, 10  $\mu$ m.

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DETAILED DESCRIPTION OF THE INVENTION

The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

C=cytosine      A=adenosine  
T=thymidine    G=guanosine

This invention provides an isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

As used herein, tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses protein (TREX) is a protein first identified as a potential tumor suppressor gene involved in tumor necrosis factor receptor (TNFR) superfamily. Furthermore, TREX is a signal modulator which bridges between TNFR and CD40-mediated signal transduction.

In an embodiment the above-described isolated nucleic acid molecule is a DNA molecule or a fragment thereof. In another embodiment the isolated DNA molecule is a cDNA molecule. In a further embodiment the DNA molecule is a genomic DNA molecule. In an embodiment the nucleic acid molecule is an RNA molecule. In another embodiment the nucleic acid molecule encodes a mammalian Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein or a functionally active fragment thereof, e.g. a motif that interacts with TRAF proteins, including but not limited to motifs such as an isoleucine zipper motif and an EXT-C domain. In an embodiment the encoded mammalian Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein is human Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-

interacting hereditary multiple extoses (TREX) protein.

5 The DNA molecules of the subject invention also include DNA molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

10 20 The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide, TREX, and as products for the large scale synthesis of the polypeptide (TREX) or fragments thereof (e.g. for the production of portions of the polypeptide encoding an isoleucine zipper motif, a hereditary multiple extoses C (EXT C) domain, or an isoleucine zipper motif and a hereditary multiple extoses C (EXT C) domain, portions which are involved in protein-protein interactions) by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide (TREX) or portions thereof which comprise an isoleucine zipper motif and/or a hereditary multiple extoses C (EXT C) domain and related

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products.

5 In an embodiment the isolated nucleic acid molecule encoding the mammalian Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein is a mouse, rat or human Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In another embodiment the isolated nucleic acid molecule  
10 encodes a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein comprising an amino acid sequence as set forth in Figures 1 and 7B (SEQ ID NO: 2). In an embodiment the isolated nucleic acid molecule encodes a mouse TREX  
15 protein. In another embodiment the isolated nucleic acid molecule encodes a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein comprising an amino acid sequence as set forth in Figures 1 and 8B (SEQ ID NO:4). In an  
20 embodiment the isolated nucleic acid molecule encodes a human TREX protein.

In an embodiment of the isolated nucleic acid molecule the encoded amino acid sequence comprises an isoleucine zipper  
25 motif and a hereditary multiple extoses C (EXT C) domain. In an embodiment the isolated nucleic acid is a fragment of the above-described nucleic acid, said fragment encoding an isoleucine zipper motif, a hereditary multiple extoses C (EXT C) domain, or an isoleucine zipper motif and a  
30 hereditary multiple extoses C (EXT C) domain. In another embodiment the Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein has substantially the same amino acid sequence as set forth in Figures 1 and 7B (SEQ ID NO: 2).  
35 In a preferred embodiment the Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein has substantially



the same amino acid sequence as set forth in Figures 1 and 8B (SEQ ID NO: 4). In another embodiment the Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein has the amino acid sequence as set forth in Figure 1 and 7B (SEQ ID NO: 2). In preferred embodiment the Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein has the amino acid sequence as set forth in Figure 1 and 8B (SEQ ID NO: 4).

This invention provides an isolated nucleic acid molecule encoding a mutant homolog of the mammalian Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein whose genetic alterations and resulting amino acid sequence(s) is set forth in Table 3, infra. In an embodiment the isolated nucleic acid molecule is a deletion mutant. In an embodiment of the deletion mutant the encoded mutant homolog comprises a tumor suppressor locus. In an embodiment of the deletion mutant the encoded mutant homolog does not comprise a tumor suppressor locus domain. In a further embodiment the above-described isolated nucleic acid molecule encoding the mammalian TREX protein comprises the genetic alterations and resulting amino acid sequence(s) as shown in Table 3, infra.

This invention provides a vector comprising the isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In an embodiment the vector is adapted for expression in a host cell which comprises the regulatory elements necessary for expression of the nucleic acid molecule in the host cell operatively linked to the nucleic acid molecule encoding the Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein so

as to permit expression of the TREX protein. In another embodiment of the vector the host cell is a eukaryotic, bacterial, insect or yeast cell. In an embodiment of the vector the eukaryotic host cell is a mammalian cell. In a further embodiment the vector is a plasmid. In another embodiment of the vector comprising the nucleic acid encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein the nucleic acid molecule is a DNA molecule. In an embodiment the DNA molecule is a cDNA molecule. In further embodiments, any of the above-described vectors are adapted for expression in a host cell which comprises the regulatory elements necessary for expression of the nucleic acid molecule in the host cell operatively linked to the nucleic acid molecule encoding the Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein as to permit expression of the TREX protein. In an embodiment of the vector, the host cell is a eukaryotic, bacterial, insect or yeast cell. In another embodiment of the vector, the eukaryotic host cell is a mammalian cell. In a further embodiment of the vector is a plasmid.

Numerous vectors for expressing the inventive proteins may be employed. Such vectors, including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses, are well known in the art. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow for the selection of transfected host cells. The markers may provide, for example, prototrophy to an auxotrophic host, biocide resistance or resistance to heavy metals such as copper. The selectable marker gene can

be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation.

5 Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. Additional elements may also be needed for optimal synthesis of mRNA. These additional elements may include splice signals, as  
10 well as enhancers and termination signals. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or  
15 homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for  
20 example the methods described above for constructing vectors in general.

These vectors may be introduced into a suitable host cell to form a host vector system for producing the inventive  
25 proteins. Methods of making host vector systems are well known to those skilled in the art.

Suitable host cells include, but are not limited to, bacterial cells (including gram positive cells), yeast  
30 cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to HeLa cells, Cos cells, CV1 cells and various primary mammalian cells. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell  
35 NIH-3T3 cells, CHO cells, HeLa cells, Ltk<sup>-</sup> cells and COS cells. Mammalian cells may be transfected by methods well known in the art such as calcium phosphate precipitation,

electroporation and microinjection.

One of ordinary skill in the art will easily obtain unique sequences from the cDNA cloned in plasmids. Such unique sequences may be used as probes to screen various mammalian cDNA libraries and genomic DNAs, e.g. mouse, rat and bovine, to obtain homologous nucleic acid sequences and to screen different cDNA tissue libraries to obtain isoforms of the obtained nucleic acid sequences. Nucleic acid probes from the cDNA cloned in plasmids may further be used to screen other human tissue cDNA libraries to obtain isoforms of the nucleic acid sequences encoding TREX as well as to screen human genomic DNA to obtain the analogous nucleic acid sequences. The homologous nucleic acid sequences and isoforms may be used to produce the proteins encoded thereby.

This invention provides a method of producing a host cell operatively linked to the nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein, which comprises growing a host cell comprising any of the above-described vectors under suitable conditions permitting production of the TREX protein and recovering the TREX protein so produced. In an embodiment the method further comprising purifying the recovered TREX protein.

This invention provides a method of producing a polypeptide having the biological activity of a protein encoded by the nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein which comprises growing any of the above-described host cells under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced. In an embodiment the method further comprises purifying the recovered polypeptide.

This invention provides a purified mammalian Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In an embodiment the purified mammalian Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein is a human TREX protein.

This invention provides a protein comprising substantially the amino acid sequence set forth in Figure 1.

This invention provides an oligonucleotide comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of an isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In an embodiment of the oligonucleotide the nucleic acid is DNA. In another embodiment of the oligonucleotide, the nucleic acid is RNA. In an embodiment the oligonucleotide comprises a nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of an isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

This invention provides an antisense oligonucleotide comprising a sequence capable of specifically hybridizing with a unique sequence included within an mRNA molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

This invention provides an antisense oligonucleotide comprising a sequence capable of specifically hybridizing with a unique sequence included within a genomic DNA

molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein.

5 This invention provides an antibody capable of binding to any of the above-described mammalian Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) proteins. In an embodiment the antibody is a monoclonal antibody. In  
10 another embodiment the antibody is a polyclonal antibody.

This invention provides a monoclonal antibody directed to an epitope of a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple  
15 extoses (TREX) protein.

Polyclonal antibodies may be produced by injecting a host animal such as rabbit, rat, goat, mouse or other animal with the immunogen of this invention, e.g. a purified mammalian  
20 TREX or a purified human TREX. The sera are extracted from the host animal and are screened to obtain polyclonal antibodies which are specific to the immunogen. Methods of screening for polyclonal antibodies are well known to those of ordinary skill in the art such as those disclosed in  
25 Harlow & Lane, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY: 1988) the contents of which are hereby incorporated by reference.

The monoclonal antibodies may be produced by immunizing for  
30 example, mice with an immunogen. The mice are inoculated intraperitoneally with an immunogenic amount of the above-described immunogen and then boosted with similar amounts of the immunogen. Spleens are collected from the immunized mice a few days after the final boost and a cell suspension is  
35 prepared from the spleens for use in the fusion.

Hybridomas may be prepared from the splenocytes and a murine

tumor partner using the general somatic cell hybridization technique of Kohler, B. and Milstein, C., Nature (1975) 256: 495-497. Available murine myeloma lines, such as those from the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852 USA, may be used in the hybridization. Basically, the technique involves fusing the tumor cells and splenocytes using a fusogen such as polyethylene glycol. After the fusion the cells are separated from the fusion medium and grown in a selective growth medium, such as HAT medium, to eliminate unhybridized parent cells. The hybridomas may be expanded, if desired, and supernatants may be assayed by conventional immunoassay procedures, for example radioimmunoassay, using the immunizing agent as antigen. Positive clones may be characterized further to determine whether they meet the criteria of the invention antibodies.

Hybridomas that produce such antibodies may be grown in vitro or in vivo using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, as the case may be, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired.

In the practice of the subject invention any of the above-described antibodies may be labeled with a detectable marker. In one embodiment, the labeled antibody is a purified labeled antibody. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. A "detectable moiety" which functions as detectable labels are well known to those of ordinary skill in the art and include, but are not limited to, a fluorescent label, a

radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase,  $\beta$ -galactosidase, fluorescein or streptavidin/biotin. Methods of labeling antibodies are well known in the art.

This invention provides a method of inhibiting TREX protein interaction with a TRAF protein comprising administering a ligand comprising an amino acid domain which binds to a EXT C domain of the TREX protein so as to inhibit binding of the TREX protein to the TRAF protein. In an embodiment the TREX protein is a mammalian protein. In a preferred embodiment, the TREX protein is a human protein.

Inhibition of the TREX protein interaction with a TRAF protein may prevent TRAF induced NF- $\kappa$ B activation. Accordingly the above-described method may be used to control cell differentiation, cell proliferation, and apoptosis (programmed cell death). Accordingly, this method would be used to treat diseases such as cancer, autoimmune diseases and inflammation by inhibiting tumor cell growth and differentiation.

As used herein ligands comprising an amino acid domain which binds to a TREX protein, which binds to a TRAF binding domain, or which block TRAF binding are defined as an amino acid molecule or fragment thereof which has an amino acid sequence complementary to a TREX protein.

This invention provides a method of inhibiting overexpression of TREX protein comprising administering any of the above-described antisense oligonucleotides which bind to an mRNA molecule encoding a human Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein so as to inhibit



overexpression of the human TREX protein.

5 In an embodiment of the above-described method inhibiting overexpression of TREX protein thereby inhibits TRAF-induced CD40 signal dependent NF-kB activation. Accordingly the above-described method may be used to control cell differentiation, cell proliferation, and apoptosis (programmed cell death). Accordingly, this method would be used to treat diseases such as cancer, autoimmune diseases and inflammation by inhibiting tumor cell growth and differentiation.

10 In another embodiment of the above-described method the ligand is an antibody capable of binding to the TREX protein. In a further embodiment of the above-described method the antibody is a monoclonal or a polyclonal antibody.

15 This invention provides a method of inhibiting growth of a tumor cell comprising blocking a TRAF interacting site of a TREX protein by administering a ligand capable of binding to the TRAF interacting site of a TREX protein.

20 In an embodiment of the above-described method, the TRAF interacting site is a hereditary multiple extoses C (EXT C) domain. In another embodiment the tumor cell growth is inhibited in vivo or in vitro. In a further embodiment the ligand is an antibody capable of binding to the TRAF interacting site of a TREX protein. In still further  
25 30 embodiments the antibody is a monoclonal or a polyclonal antibody.

This invention provides a pharmaceutical composition comprising an amount of any of the above-described  
35 oligonucleotides effective to prevent overexpression of a TREX protein and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

This invention provides a pharmaceutical composition comprising an amount of any of the above-described antibodies effective to block binding of a TREX protein to a TRAF protein and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

This invention provides a method of administering the above-described pharmaceutical compositions comprising an amount of any of the above-described ligands, oligonucleotides or antibodies which are determined to be potentially therapeutic, wherein the administration is intravenous, intraperitoneal, intrathecal, intralymphatical, intramuscular, intralesional, parenteral, epidural, subcutaneous; by infusion, liposome-mediated delivery, aerosol delivery; topical, oral, nasal, anal, ocular or otic delivery.

The present invention also provides a pharmaceutical composition comprising an effective amount of any of the above-described ligands, oligonucleotides or antibodies which are determined to be potentially therapeutic and a pharmaceutically acceptable carrier. In the subject invention an "effective amount" is any amount of the above-described ligands, oligonucleotides or antibodies which are determined to be potentially therapeutic, which, when administered to a subject suffering from a disease or abnormality against which the above-described ligands, oligonucleotides or antibodies which are determined to be potentially therapeutic, are effective, causes reduction, remission, or regression of the disease or abnormality. In the practice of this invention the "pharmaceutically acceptable carrier" is any physiological carrier known to those of ordinary skill in the art useful in formulating pharmaceutical compositions.

In one preferred embodiment the pharmaceutical carrier may be a liquid and the pharmaceutical composition would be in

the form of a solution. In another equally preferred embodiment, the pharmaceutically acceptable carrier is a solid and the composition is in the form of a powder or tablet. In a further embodiment, the pharmaceutical carrier is a gel and the composition is in the form of a suppository or cream. In a further embodiment the compound may be formulated as a part of a pharmaceutically acceptable transdermal patch.

10 A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidone, low melting waxes and ion exchange resins.

25 Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially

containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by for example, intramuscular, intrathecal, epidural, intraperitoneal or subcutaneous injection. Sterile solutions can also be administered intravenously. The compounds may be prepared as a sterile solid composition which may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium. Carriers are intended to include necessary and inert binders, suspending agents, lubricants, flavorants, sweeteners, preservatives, dyes, and coatings.

The above-described ligands, oligonucleotides or antibodies which are determined to be potentially therapeutic can be administered orally in the form of a sterile solution or suspension containing other solutes or suspending agents, for example, enough saline or glucose to make the solution isotonic, bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like.

The above-described ligands, oligonucleotides or antibodies which are determined to be potentially therapeutic can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include

solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular ligands, oligonucleotides or antibodies in use, the strength of the preparation, the mode of administration, and the advancement of the disease condition or abnormality. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

This invention provides a method of treating an abnormality in a subject, wherein the abnormality is alleviated by the inhibition of binding of a TREX protein and a TRAF protein which comprises administering to the subject an effective amount of the above described pharmaceutical composition effective to block binding of the TREX protein and the TRAF protein in the subject, thereby treating the abnormality in the subject. In an embodiment the TRAF protein is TRAF2, TRAF3 or TRAF 5. In a preferred embodiment the abnormality is cancer, a hereditary multiple extosis or an autoimmune disease. In a further preferred embodiment the cancer is colon cancer, gastric cancer, human squamous cell carcinoma, prostate carcinoma, breast cancer, or papillary bladder cancer.

This invention provides a method of treating an abnormality in a subject, wherein the abnormality is alleviated by the inhibition of overexpression of a TREX protein which comprises administering to the subject an effective amount of the above-described pharmaceutical composition effective to inhibit overexpression of the TREX protein, thereby

treating the abnormality in the subject. In a preferred embodiment the abnormality is cancer, a hereditary multiple extosis or an autoimmune disease. In a further preferred embodiment the cancer is colon cancer, gastric cancer, human head and neck squamous cell carcinoma, prostate carcinoma, breast cancer, thyroid cancer, esophageal cancer, lung cancer, colorectal cancer, ovarian cancer, papillary bladder cancer, osteosarcoma, chondrosarcoma, liposarcoma, giant cell tumor, Ewing sarcoma, and other malignant tumors.

This invention provides a method of screening for a chemical compound which inhibits TREX protein and TRAF protein binding comprising: (a) incubating the chemical compound with a TREX protein and a TRAF protein; (b) contacting the incubate of step (a) with an affinity medium under conditions so as to bind a TREX protein-TRAF protein complex, if such a complex forms; and (c) measuring the amount of the TREX protein-TRAF protein complex formed in step (b) so as to determine whether the compound is capable of interfering with the formation of the complex between the TREX protein-TRAF protein.

Additional methods for an assay to screen for drugs which inhibit the TREX-TRAF binding which are known to one of ordinary skill in the art include but are not limited to the two-hybrid screening system using yeast and mammalian cells (Fields, S. and O. Song, Nature, 340:245-246, 1989, the contents of which are hereby incorporated by reference).

In the above-described methods of screening for a chemical compound which inhibits TREX protein and TRAF protein binding association conditions, including but not limited to low salt, pH, or temperature may be used to compare the amount of TREX-TRAF complex formed without incubation with the compound.

In an embodiment the TRAF protein is TRAF2, TRAF3 or TRAF 5.

In a preferred embodiment the compound may be a CD40 receptor ligand or a CD40 antibody.

In a preferred embodiment of the above-described methods, the molecule is a peptide or a fragment thereof which comprises a TRAF binding domain. In further embodiments the TRAF protein is TRAF2, TRAF3 or TRAF 5.

This invention provides a method of preventing inhibition of activation of a CD40 signal-dependent NF-kB activation comprising administering any of the above-described antisense oligonucleotides which bind to an mRNA molecule encoding a human Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein so as to prevent inhibition of activation of CD40 signal-dependent NF-kB activation.

This invention provides a method of preventing inhibition of activation of a CD40 signal-dependent NF-kB activation comprising administering a ligand comprising an amino acid domain which binds to a EXT C domain of the TREX protein so as to inhibit binding of the TREX protein to the TRAF protein, thereby preventing inhibition of activation of a CD40 signal-dependent NF-kB activation.

In a preferred embodiment of the above-described method the ligand is peptide or a fragment thereof which comprises a TRAF binding domain.

This invention provides a method of detecting a predisposition to cancer which comprises detecting of a genetic alteration in a nucleic acid encoding TREX protein in the sample from the subject. In a preferred embodiment of the above-described method the mutation is a silent point mutation or a missense point mutation. In another preferred embodiment of the above-described method the genetically altered nucleic acid encoding TREX protein is detected by

contacting the nucleic acid from the sample with a TREX nucleic acid probe under conditions permitting the TREX nucleic acid probe to hybridize with the nucleic acid from the sample, thereby detecting the genetic alteration in the nucleic acid encoding TREX protein in the sample.

Methods of detecting genetic alterations in nucleic acid molecules are well known to one of ordinary skill in the art and include but are not limited to methods such as single strand conformation polymorphism detection, RNase protection assay, and PCR direct sequencing. As used herein, genetic alterations in nucleic acid molecules which may be detected include point mutations, deletions, translocations, and insertions.

In other preferred embodiments the cancer is colon cancer, gastric cancer, human head and neck squamous cell carcinoma, prostate carcinoma, breast cancer, thyroid cancer, esophageal cancer, lung cancer, colorectal cancer, ovarian cancer, papillary bladder cancer, osteosarcoma, chondrosarcoma, liposarcoma, giant cell tumor, Ewing sarcoma, and other malignant tumors. In another preferred embodiment of the above-described method the TREX nucleic acid probe comprises a nucleic acid molecule of at least 15 nucleotides which specifically hybridizes with a unique sequence included within the sequence of an isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In an embodiment of the TREX nucleic acid probe the nucleic acid is DNA. In another embodiment of the TREX nucleic acid probe the nucleic acid is RNA.

This invention provides a TREX nucleic acid probe comprising a sequence capable of specifically hybridizing with a unique sequence included within the above-described isolated DNA molecule encoding a Tumor necrosis factor Receptor-



Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In an embodiment the nucleic acid probe comprises a nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In a further embodiment the TREX is mammalian protein. In an embodiment the mammalian TREX protein is mouse protein. In a preferred embodiment the mammalian TREX protein is human protein.

This invention provides a TREX nucleic acid probe comprising a sequence capable of specifically hybridizing with a unique sequence included within the above-described isolated mRNA molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In an embodiment the nucleic acid probe comprises a nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In a further embodiment the TREX is mammalian protein. In an embodiment the mammalian TREX protein is mouse protein. In a preferred embodiment the mammalian TREX protein is human protein.

This invention provides a TREX nucleic acid probe comprising a sequence capable of specifically hybridizing with a unique sequence included within the above-described isolated genomic DNA molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In an embodiment of the method the mutation comprises a portion of

a tumor suppressor locus. In an embodiment the nucleic acid probe comprises a nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the isolated nucleic acid molecule encoding a Tumor necrosis factor Receptor-Associated Factor (TRAF) protein-interacting hereditary multiple extoses (TREX) protein. In a further embodiment the TREX is mammalian protein. In an embodiment the mammalian TREX protein is mouse protein. In a preferred embodiment the mammalian TREX protein is human protein.

This invention provides a method of diagnosing cancer in a subject which comprises: a) obtaining DNA from the sample of a subject suffering from cancer; b) performing a restriction digest of the DNA with a panel of restriction enzymes; c) separating the resulting DNA fragments by size fractionation; d) contacting the resulting DNA fragments with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a genetically altered nucleic acid molecule encoding a TREX protein, wherein the nucleic acid is labeled with a detectable marker; e) detecting labeled bands which have hybridized to the nucleic acid probe in step (d), wherein the sequence of a genetically altered nucleic acid molecule encoding a TREX protein creates a unique band pattern specific to the DNA of subjects suffering from cancer; f) preparing DNA obtained from a sample of a subject for diagnosis by steps (a-e); and g) comparing the detected band pattern specific to the DNA obtained from a sample of subjects suffering from cancer from step (e) and the DNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to cancer if the patterns are the same.

As used herein, genetic alterations in nucleic acid molecules which may be detected include point mutations,

deletions, translocations, and insertions.

In an embodiment of the above-described method the size fractionation in step (c) is effected by a polyacrylamide or agarose gel. In another embodiment of the method the detectable marker is radioactive isotope, enzyme, dye, biotin, a fluorescent label or a chemiluminescent label. In a preferred embodiment of the above-described method, cancer associated with the expression of a mutated TREX protein is diagnosed. In further preferred embodiments of the above-described method the cancer is colon cancer, gastric cancer, human head and neck squamous cell carcinoma, prostate carcinoma, breast cancer, thyroid cancer, esophageal cancer, lung cancer, colorectal cancer, ovarian cancer, papillary bladder cancer, osteosarcoma, chondrosarcoma, liposarcoma, giant cell tumor, Ewing sarcoma, and other malignant tumors.

This invention provides a method of diagnosing cancer in a subject which comprises: a) obtaining RNA from the sample of the subject suffering from cancer; b) separating the RNA sample by size fractionation; c) contacting the resulting RNA species with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mutated TREX protein, wherein the sequence of the nucleic acid molecule encoding the mutated TREX protein is labeled with a detectable marker; d) detecting labeled bands which have hybridized to the RNA species to create a unique band pattern specific to the RNA of subjects suffering from cancer; e) preparing RNA obtained from a sample of a subject for diagnosis by steps (a-d); and f) comparing the detected band pattern specific to the RNA obtained from a sample of subjects suffering from cancer from step (d) and the RNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to cancer if the patterns are the same. In an embodiment of the

method the size fractionation in step (c) is effected by a polyacrylamide or agarose gel. In another embodiment of the method the detectable marker is radioactive isotope, enzyme, dye, biotin, a fluorescent label or a chemiluminescent label. In a preferred embodiment of the above-described method, cancer associated with the expression of a mutated TREX protein is diagnosed. In further preferred embodiments of the above-described method the cancer is colon cancer, gastric cancer, human squamous cell carcinoma, prostate carcinoma, breast cancer, or papillary bladder cancer.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

#### FIRST SERIES OF EXPERIMENTS

Tumor necrosis factor (TNF) receptor-associated factor (TRAF) proteins contribute to signal transduction induced by TNF receptor family signaling. TRAF3 cloned as binding protein to the cytoplasmic domain of CD40, a member of TNF receptor superfamily, is believed to be involved in signaling pathway induced by CD40, Lymphotoxin (LT)  $\beta$  receptor, CD30 ligation (1-7). Here molecular cloning of a novel TRAF-interacting protein named as TREX because of TRAF-interacting EXT (hereditary multiple exostoses) gene family protein is reported. TREX has a highly homologous sequence to the EXT gene family, a candidate of tumor suppressor gene (20-22). TREX strongly interacts with TRAF2 and TRAF3, and TREX and TRAF protein colocalize in mammalian cells. Moreover, overexpression of TREX inhibited NF- $\kappa$ B activity induced by TRAF-mediated signaling. These findings indicate that TREX and the other EXT gene family proteins can function as a mediator in receptor signaling and could

be involved in tumorigenesis.

## EXPERIMENTAL DETAILS

### METHODS AND MATERIALS

#### **Two-hybrid screening**

Two-hybrid screening was performed in yeast L40 (MATa) strain cells with plasmid pBTM116 containing human TRAF3 (amino acids 82-543) subcloned in frame with the LexA as a bait and a mouse embryo cDNA library cloned into pVP16 as described previously (36). In order to obtain the clones containing cDNA encoding protein which binds specifically to TRAF3, clones that formed on histidine-deficient media and produced a blue reaction product with X-gal in filter assays (37) were cured of the LexA-TRAF3 plasmid by growing cells in tryptophan-containing medium, and then mated against a panel of yeast strains NA87-11A (MAT $\alpha$ ) containing plasmid pBTM116 that produce LexA fusion protein with lamin, Fas and CD40. Mated cells were selected for growth in medium lacking tryptophan and leucine, and subsequently tested for the ability to trans-activate a lacZ reporter gene by growing cells on histidine-deficient media and a  $\beta$ -Gal colometric assay (37).

#### **Northern blot analysis**

Human and mouse Multiple Tissue Northern Blots (Clontech) were probed with human and mouse TREX cDNA, respectively.

#### **Plasmid construction and transfection**

Full length coding regions of TRAFs, TREX and their mutants were amplified by PCR and subcloned into FLAG-tagged pCR3.1 or myc-tagged pcDNA3.1 (Invitrogen). Mouse CD40 and CD40L were also amplified by PCR and subcloned into pMIKHygB. 293 cells and 293 T cells were transfected by standard calcium

phosphate coprecipitation method. COS cells were transfected by use of FuGENE 6 (Boehringer Mannheim).

#### **Production of anti-TREX, immunoprecipitation and western blot analysis**

Rabbit anti-TREX polyclonal antibody raised against a recombinant Glutathion S-transferase-fused mouse TREX protein. 293T cells ( $2 \times 10^6$  cells) were transfected with the indicated plasmids. After transfection (40 hours), cell lysates were prepared in Lysis buffer (20 mM Tris (pH 7.6), 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA (pH 8.0), 10  $\mu$ g/ml of aprotinin, 10  $\mu$ g/ml of leupeptin, 5 mM Benzamidine and 1 mM PMSF) and incubated with indicated antibodies and 25  $\mu$ l of 50% slurry of protein G-Sepharose. Immunoprecipitates were detected by Western blot analysis using the indicated antibody. To detect endogenous TREX protein, cell lysates of human colon carcinoma cell line KM12L4 were immunoprecipitated with anti-TREX antibody and detected by Western blot analysis using anti-TREX antibody.

#### **Immunohistochemistry**

COS7 cells were transfected with TRAF3 or myc-tagged TREX. After transfection (40 hours), cells were fixed with methanol. For detection of TREX protein, Anti-myc antibody (9E10, BIOMOL) and Phycoerythrin-anti-mouse IgG (Chemicon) were used for 1st and 2nd antibody, respectively. For detection of TRAF protein, anti-TRAF3 antibody (Santa Cruz) and FITC-anti-rabbit IgG (Santa Cruz) were used for 1st and 2nd antibody, respectively.

#### **Reporter gene assay**

293 cells ( $1 \times 10^6$  cells) were transfected with NF- $\kappa$ B-dependent reporter gene (pKbtkLuc), the indicated plasmids and pRL-CMV (Promega) for normalization of

transfection efficiency as described previously (2). After transfection (40 hours), the cell lysates were prepared and luciferase activity measured using Dual-luciferase reporter assay system (Promega).

## EXPERIMENTAL RESULTS AND DISCUSSION

TNF receptor-associated factor (TRAF) protein family members have been reported to contribute to TNF receptor-initiated signaling through direct binding to the cytoplasmic region of receptors, resulting in the activation of many signaling molecules such as transcription factor NF- $\kappa$ B, mitogen-activated protein kinase (MAPK), although TRAF1 and TRAF4 have not been implicated clearly (2, 8-13). Overexpression of TRAF2 activates NF- $\kappa$ B and JNK/SAPK via NF- $\kappa$ B-inducing kinase (NIK)-dependent pathway and -independent pathway, respectively (14-16). TRAF5 activates NF- $\kappa$ B and TRAF6 activates NF- $\kappa$ B and ERK/MAPK pathway (2, 9-12). Although TRAF2 is implicated to be required for protection against TNF-induced apoptosis via NF- $\kappa$ B-independent pathway (17, 18), TRAF5 or TRAF6 could act to activate NF- $\kappa$ B pathway in place of TRAF2. These observations suggest that action of TRAF proteins seem to be regulated properly in response to each receptor signaling for the expression of receptor functions. On the other hand, overexpression of TRAF3 has been demonstrated to suppress the activation of NF- $\kappa$ B and ERK/MAPK induced by CD40 crosslinking (2, 8). TRAF3 is implicated to be required for postnatal development and T-dependent immune responses (19), but no plausible signaling pathways or molecules via TRAF3 which lead to explain these biological functions were reported so far, in turn, the specificity and function of TRAF3-mediated signaling are still unclear.

Analyzing the signaling molecules downstream of TRAF3 would provide an understanding of the function of TRAF3 and its specificity. To identify the signaling molecules which

specifically bind to TRAF3, two-hybrid screening of a mouse embryo cDNA library was performed using TRAF3 (amino acids 82-543) as a bait. In this screening, multiple cDNA clones encoding several kinds of proteins were identified by sequencing. One clone among these positive clones, showed a putative isoleucine zipper motif in its sequence (Fig. 1a). On the basis of a partial sequence, marathon PCR amplification and 5'-RACE methods were carried out, and a mouse full length sequence with an open reading frame of 2,757 bp, which encodes a 918 amino acid peptide, was obtained (Fig. 1a). Human full length cDNA with an open reading frame of 2,760 bp, which encodes a 919 amino acid peptide with 96.8 % identity to the mouse sequence, was also identified by screening of a human fetal brain cDNA library and the 5'-RACE method (Fig. 1a). A BLAST data base search revealed that the C-terminal region of these clones shows significant homology to hereditary multiple exostoses (EXT) gene family proteins such as EXT1, EXT2, EXTL1, EXTL2 and C. elegans rib-2 (Fig. 1b) (20-25). Therefore, this new gene was designated as TREX (for TRAF-interacting EXT gene family protein). Based on homology searches among EXT family proteins including TREX, permitted designating the highly homologous C-terminal regions as EXT domains, which are divided into two domains, EXT-N and EXT-C domains (Fig. 1c, d). These new conserved regions might function as signaling mediators by protein-protein interaction. Surprisingly, human and mouse TREX have significant homology to C. elegans rib-2 (Fig. 1 c, d) in not only the EXT domain but the region between the EXT-N and the EXT-C domains (33 %, data not shown). This observation suggests that TREX protein plays a critical role in development beyond species.

Next the expression of TREX mRNA and protein was examined. Northern blot analysis revealed about 7.0 kilobases transcript of TREX expressed in various tissues, with high expression in brain, heart, skeletal muscle (Fig. 1e). To examine the endogenous TREX protein in mammalian cells, cell



lysates of human colon carcinoma cell line KM12L4 were immunoprecipitated with either a normal rabbit IgG or a rabbit anti-TREX antibody. Anti-TREX antibody detected a specific band at about 107 kDa, which is consistent with the predicted molecular weight of full length TREX (Fig. 1f).

As TREX has cloned as TRAF3-binding protein, the binding specificity to TRAF family proteins was examined. The 293T cells were transfected with TREX and TRAF expression plasmids. Coimmunoprecipitation experiments indicated that not only TRAF3 but also TRAF2 strongly and TRAF5 weakly binds to TREX (Fig. 2a). This observation leads to the consideration that TRAF proteins interact with TREX through TRAF domain, which is comparatively conserved among TRAF proteins, and that TREX and TRAF protein should colocalize in the cells. To examine the localization of TREX protein and TRAF3 protein, COS7 cells were transfected with TREX or TRAF3 expression plasmids. TRAF3 protein localized in cytoplasm, especially the region outside of the nuclear membrane, and TREX also localized around the nuclear membrane (Fig. 2b). These results suggest that TREX and TRAF proteins are physically associated in mammalian cells.

The interaction of TREX and TRAF2 or TRAF3 indicated that TREX could be involved in TRAF-mediated signaling. Therefore, whether the expression of TREX protein could affect NF- $\kappa$ B activation induced by several stimulation was tested. 293 cells were transfected with TREX with CD40 and CD40 ligand in the presence of a NF- $\kappa$ B-specific reporter gene. As shown in Fig. 3, CD40 signal-dependent NF- $\kappa$ B activation was inhibited by overexpression of TREX in a dose dependent manner, indicating that TREX could contribute to NF- $\kappa$ B pathway induced by CD40 ligation. Next, applicant examined whether TREX is involved in NF- $\kappa$ B activation mediated TRAF2 or not.

Overexpression of TREX upregulated TRAF2-induced NF- $\kappa$ B

activation (Fig. 4). These results suggest that TREX acts as a negative regulator of NF- $\kappa$ B pathway by direct interaction with TRAF2 in TNF receptor type II signaling. TRAF-interacting proteins TANK/I-TRAF and TRIP proteins, which inhibit NF- $\kappa$ B activity induced by TNF receptor family stimulation, were cloned by two-hybrid screening (26-28). TRIP protein was proposed to be regulated by switching with antiapoptotic protein such as c-IAP in response to the signals leading to cell activation or cell death (26). However, as the biological function of these proteins in TRAF-mediated signaling is still unknown, it is important to further analyze the activity of several signaling molecules.

Demonstrated here is the identification of a novel TRAF-interacting protein, TREX, and the contribution of TREX protein in CD40/TNF receptor type II signaling mediated by TRAF family. Furthermore, the sequence of this new protein TREX revealed a high homology to the EXT gene family and novel domains named EXT-N and EXT-C domains. This conserved sequence in the EXT domain suggests that the EXT domain might contribute to protein-protein interaction. Whether the EXT domain of the other EXT gene family proteins is involved in protein-protein interaction or not is now under investigation.

EXT gene family proteins, EXT1 and EXT2 have been cloned by positional cloning on the basis of linkage analysis in informative exostoses families (20-22). Some mutation was found in these genes, suggesting these genes should be candidate genes responsible for EXT (20-22, 29-31). Three loci have been localized. The EXT1 and EXT2 were localized on chromosome 8q24.1, 11p11-13, respectively (20, 32, 33), and the third gene EXT3 on 19p was not identified (34). Also identified was the chromosomal localization of human TREX on chromosome 8p11-12 (Shao et al., submitted), excluding TREX as a candidate gene for EXT3. It is important to investigate whether TREX could be responsive to EXT or EXT-related

diseases. EXT family protein has been suggested to be a tumor suppressor gene because previous reports showed that multiple mutation in chondrosarcoma from sporadic tumors and tumors derived from malignant degeneration of exostoses (31, 35). Also identified was some infrequent mutation in TREX gene in some tumors (Shao et al., submitted), suggesting TREX might contribute to prevention of abnormal development such as transformation and tumorigenesis. The mutation of TREX gene in many kinds of tumor samples is being surveyed.

Not only mammals but also species such as *C.elegans* which lack bone in their body have homologous genes to the EXT gene family according to EST database search (25), suggesting that the EXT family proteins play an important role in development except bone development. A TREX-knockout mouse and rib-2-knockout *C. elegans* are being made. Knockout of EXT gene family genes in these species will facilitate an understanding of their function and their importance during development.

Five EXT gene family proteins were identified but the function of these gene products has been unknown. In this study, it is shown for the first time that an EXT family protein, TREX, acts as a signaling molecule mediating TNF receptor superfamily (Figs. 3,4). Also shown is that the EXT-domain of TREX interacts with TRAF proteins, which mediate receptor signaling through direct binding. These findings imply that the other EXT proteins could act as signaling mediators in receptor signaling. As TREX and the other EXT family proteins are easily thought to be involved in receptor signaling, the development of inhibitor(s) of signaling cascades related to TREX or the other EXT family proteins will be used to design drugs to treat many diseases including cancer.

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Second Series of Experiments

Hereditary multiple exostoses (EXT) is an autosomal dominant disorder characterized by short stature and the development of multiple bone tumour (1-3). Three genetic loci have been identified by genetic linkage analysis at chromosome 8q24.1 (EXT1) (4), 11p11-13 (EXT2) (5) and 19p (EXT3) (6). The putative tumour suppressor gene EXT1 and EXT2 were identified and characterized (7,8). Recently, two EXT-like genes, EXTL1 (9) and EXTL2 (10) have also been identified. EXTL1 and EXTL2 were mapped to chromosome 1p36.1 and 1p11-12, respectively, a region that frequently deleted in various tumour types. Previously reported was the isolation of a novel member of EXT gene family, designated TREX from mouse (11). Reported here is the isolation of TREX from human and located it at chromosome 8p11-12 by fluorescence in situ hybridization, a region that also frequently deleted in various tumours. In preliminary screens, TREX alterations were observed in some human cancers. This gene, TREX, therefore, may be a novel member of EXT gene family and may be a potential candidate which appears to be associated with the oncogenesis of multiple human genes.

Hereditary multiple exostoses (EXT) is an inherited multiple disorder characterized by the presence of exostoses, bony outgrowth capped by cartilage and with the most serious complication of chondrosarcomas or osteosarcomas (1-3). EXT1 and EXT2 were cloned (7, 8) and shown to harbor mutations in affected members of multiple exostoses families, defining two candidates as the genes responsible for multigene family of proteins with potential tumour suppressor activity. Recently, another two members of EXT-like genes, EXTL1 and EXTL2 were also identified (9, 10). Both genes were mapped to the short arm of chromosome 1, in bands 1p36 and 1p11-12, respectively, a region that frequently loss of heterozygosity in breast (12-13), gastric cancer (14), colorectal polyps (15), multiple endocrine neoplasia (16),

and cervical carcinoma (17). Nevertheless, chromosome localization of EXTL1 and EXTL2 exclude them as candidates for EXT3. However, EXTL1 and EXTL2 may play a role in those cases of multiple exostoses that cannot be linked to chromosome 8, 11 or 19. It is also possible that EXTLs might function as tumor suppressors in an entirely different cell type, due to their striking difference of chromosome locations. Therefore, searching for additional members of EXTL gene family in man and other species will be very important.

A novel member of multiple exostoses gene family was previously isolated and characterized by yeast-two hybrid approaches from mouse, which is also a novel component of TRAF signal complex, named mTRES (mouse TRAF-interaction EXT protein) (11). To identify potential coding sequences of human TRES, a 500bp of mouse cDNA which does not show homology to EXT gene family was used to screen a human adult brain cDNA library (Clontech) at low stringency condition, two overlapping positive clones were identified. Clone 1, contains an insert size of 1614bp with a partial open reading frame of 1590 (530 amino acids) followed by a stop codon and a 24bp 3'-untranslated region. Clone 2 contains an insert size of 1430bp with 118bp overlapping with Clone 1 at the 3'-untranslated region, resulting in 2926bp of the total cDNA sequence. This cDNA sequence was used to search the GenBank using BLAST search program and demonstrated a near identity and overlapping with human chromosome 8 BAC clone CIT987SK-2A8 (HSU96629, NCBI sequence ID g2341008, briefly as BAC 8). This clone was obtained and a complete sequence determined. To obtain cDNA covering additional portions of the gene a PCR-based method was used. Primers were designed from the sequence of BAC 8. PCR of a randomly primed, Jurkat total RNA with these primers produced multiple, specific bands of different sizes, which were individually cloned to yield the cDNA clones. The longest clone contains a 1197bp insert. Sequencing revealed that this clone overlapped with

the cDNA clone 1 from brain cDNA library by 51 nucleotides at the 5' direction. To extend the hTREX to a full-length cDNA sequence, a modification of the 3' and 5'-rapid amplifications of cDNA ends (RACE) were performed, producing a series of overlapping RACE products which extended the cDNA sequence 637 base pairs in the 5' direction and 1527 bp in the 3' direction. The combination of cDNA isolation from cDNA library, PCR extension and RACE extension resulted in the complete sequence of the hTREX candidate gene of 6236 bp. The whole cDNA sequence was sent to GenBank (the accession number is AF083551 for human TREX). The longest continuous coding region is 2760bp starting at nucleotide 638, and is preceded by 6 in frame stop codons upstream. The predicted 5' and 3'-untranslated region (UTR) is unusually long as compared with the 5' and 3' UTR sequences which have been found in some proto-oncogenes as well as human transforming growth factor- $\beta$  (18).

The cDNA sequence is identical to BAC 8 which had previously been mapped to chromosome 8p. To further determine the finest chromosome location of TREX, cDNA clone containing the whole open reading frame was purified and hybridized to metaphase chromosome spreads using fluorescence in situ hybridization (FISH). This analysis positioned TREX on chromosome 8p11-12 (Figure 5), a region of the genome is frequently deleted in tumors from human squamous cell carcinomas of the head and neck (SCCHN) (19), prostate carcinomas (20), breast cancers (21), papillary bladder cancers (22) and colon cancers (23), and is thus believed to contain one or more tumor suppressor loci.

To further characterize the hTREX gene and to determine the intron/exon boundaries for mutational analysis, hTREX sequences were compared to BAC 8 genomic sequences. The TREX gene totally consists of 7 exons. The exact intron and exon sizes have been determined. All exon-intron splice junctions conform to the eukaryotic 5'-donor and 3'-acceptor consensus

splice junction sequence GT-AG (24) (Table 1). Of the 6 splice junctions, 3 occurred between codons, and 3 interrupted codons.

- 5 The fact that the TREX candidate gene showed significant similarity with EXT gene family and mapped within the region deleted in a variety of tumor types, strongly suggests that it is therefore a novel member of the EXT gene family as

**Table 1. The sizes and junction sequences for exon/introns of hTREX**

No.	Size (bp)		Sequences at exon-intron junction	
	Exon	Intron	3' splicing acceptor	5' splicing donor
1	71	11800		AGCCG <u>gt</u> aggac
2	94	2033	aaatc <u>ag</u> GAGAG	ACATG <u>gt</u> gaggga
3	2623	13035	tttcag <u>gc</u> CTG	TCATG <u>gt</u> aatag
4	128	6167	ataca <u>ag</u> GTGGT	TTCCG <u>gt</u> gagag
5	145	5421	tttca <u>ag</u> GGTGT	ACAAG <u>gt</u> aagaa
6	129	7433	ctgac <u>ag</u> TATTA	TCAAG <u>gt</u> gaggt
7	3029		tttcag <u>ag</u> GTGAC	

- 10 well as a potential candidate for several tumor phenotypes. To facilitate the search for mutations of whole open reading form of TREX, 5 sets of primer pairs for PCR amplification and 12 sequencing primers were selected from the flanking intronic or exonic sequences (Table 2).

**Table 2. Primers for PCR amplification and Sequencing of human TREX**

Exon 3	5' forward primer	5' TTATGGCGAGTGACCCGACGTG 3'
	3' reverse primer	5' TTGCTAAAGTGAAGGAAGTTGG 3'
	sequencing primers	5' ACCCGACGTGATCTGG 3'
	(forward)	5' AAGAGCTCCTGCAGCTGG
		5' TTCTCGTTGCCCTCTCAC 3'
		5' ATCATCAATCTGTCACG 3'
		5' ACTACGATGACCGGATC 3'
		5' TTCCCTACCAGGACATGC 3'
		5' AACATGGCTGACAACG 3'
		5' TATTGGTGGTGGAGCTGG 3'
Exon4	5' forward primer	5' AATCCAGCCATGGTCTCCTTGG 3'
	3' reverse primer	5' AGTCGATGCCATTATTACCAGC 3'
	sequencing primers	5' TTCCTTCCTCATCACAG 3'
	(forward)	
Exon 5	5' forward primer	5' AGGTCTGTGTATGCACTTGTG 3'
	3' reverse primer	5' AGTCGATGCCATTATTACCAGC 3'
	sequencing primers	5' TTCAAGGTGTGGAGAG 3'
	(forward)	
Exon 6	5' forward primer	5' TTGGCTGAAAGCCAACAACCTG 3'
	3' reverse primer	5' AACATGCACGCATCCACAGC 3'
	sequencing primers	5' TTGTAACACAGCATGTGG 3'
	(forward)	
Exon 7	5' forward primer	5' GGTTCTGTCACTATTAGCTGGG 3'
	3' reverse primer	5' TTCCTCCCTCTGCTCATCCTC 3'
	sequencing primers	5' TTCCCACTCTGTCTCTC 3'
	(forward)	

Genetic alterations of TREX were further analyzed in breast cancers as wells as various tumors in which frequent LOHs were observed on 8p. A total of 315 primary tumors originated from a variety of organs and 14 cancer cell lines were analyzed. Mutations in the entire coding regions as well as surrounding intron-exon boundaries, were analyzed, but no somatic mutations were detected. In Case 9, a thyroid cancer patient, had a 9-bp insertion in her constitutional DNA. This 9-bp has been inserted at a direct repeat with a T as a spacer: 5'-GATGAGGC-T-GATGAGGC-A-3' resulting 5'-GATGAGGC-T-GATGAGGC-T-GATGAGGC-A-3', and amino acid sequence would change from Asp-Glu-Ala-Asp-Glu-Ala to Asp-Glu-Ala-Asp-Glu-Ala-Asp-Glu-Ala.

A G to A transition at the third nucleotide of codon 171 was also observed in one lung cancer cell line EBC-1. This base substitution does not change amino acid coding. Since the constitutional DNA of this cell line was not available, it is not possible to determine whether or not this base substitution occurred somatically. Although other 328 tumors did not harbor this base substitution, the possibility of a rare polymorphism cannot be excluded. A C to T transition at codon 605 was found only in two of 329 tumors. Again this base substitution does not affect amino acid coding. Constitutional DNAs of the patients of these two tumors also harbored this base substitution. 50 normal volunteers were also analyzed but none of them had this base substitution. However, this base substitution is thought to be a rare polymorphism rather than germline mutation. Besides these alterations, three polymorphisms were found: a polymorphism with no amino acid change in exon 3, at codon 409, and two polymorphisms in introns 4 and 5. These results are summarized in Table 3.

Table 3. Genetic alterations detected in hHPV

	Position <sup>a</sup>	Alteration	Predicted effect
5	Exon 3 55	9bp insertion <sup>b</sup>	3 amino acid insertion
10	Exon 3 171	CCG/CCA	silent <u>LL</u>
15	Exon 3 409	CCA/CCG	polymorphism (CCA/CCG 15/13)
	Exon 3 605	AAC/AAT	polymorphism <u>LL</u> (AAC/AAT 100/0)
20	Intron 4 +36	A/G	polymorphism (A/G-29/17)
	Intron 5 -30	G/C	polymorphism (G/C-16/30)

a) In exons, positions were indicated by the codons.

b) In introns, + and - indicate downstream from the donor site and upstream from the acceptor site, respectively. This 9-bp insertion was observed in the constitutional DNA of one thyroid cancer (papillary carcinoma) patient.

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METHODS AND MATERIALS

5 cDNA library screening. A 500bp of cDNA insert of mouse TREX was purified from a digest of pBluescript DNA by agarose gel electrophoresis, labeled by random priming, and used to screen  $1 \times 10^{10}$  plaques of an oligo(dT) + random primed human adult brain cDNA library (Clontech) at reduced stringency condition. Inserts from the clones identified in this way were transferred into pBluescript plasmids.

10 RT-PCR cDNA extension. Total RNA prepared from Jurkat cells was used for in vitro transcription. About 10  $\mu$ g of total RNA was used as a template in a 25  $\mu$ l RT reaction containing 40  $\mu$ g of hexamer random primers. 10  $\mu$ l of RT product was then used as a template in a 100  $\mu$ l PCR reaction. Thirty  
15 cycles of amplification (1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C) were performed, and the products were analyzed on agarose gels. Products with unique sizes were produced from several primers. Individual products were excised from  
20 the gel, purified form QIAquick Gel Extracriion Kit (QIAGEN), and cloned into the PCR II vector (Invitrogen).

25 3' and 5'-RACE-Ready™ cDNAs from human brain and muscle were obtained from Clontech. PCR reactions were performed according to the manufacturer's protocol using the primers supplied with the cDNAs. PCR products were cloned to pCR II vectors as describe above.

30 DNA sequencing and analysis. DNA sequences were determined using ThermoSequenase (Amersham),  $\alpha$ - $^{32}$ P-ddNTP labeling, and autoradiographic detection. Complete sequences for both sense and antisense strands were determined for the cDNA. DNA and protein sequence analysis and database searches were performed using MacVector™ sequence analysis software  
35 (Osford Molicular Group) and by BLAST program.



## Fish Analysis

Metaphase or prophase spreads were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes of a normal healthy female volunteer (Inazawa et al., 1994) (25). Slides were denatured at 75°C for 3 min in 70% formamide/2XSSC (0.3M NaCl, 0.03M sodium citrate, pH7), immersed in 70% ethanol at -20°C, and dehydrated in 100% ethanol. Two-color FISH, using pBSIISK(+)-TREX, a plasmid clone which contains TREX cDNA and RMC08L009 (pJM128), a plasmid clone which contains chromosome 8 centromere sequence (Donlon et al., 1986) (26), was performed essentially as described previously (Inazawa et al., 1993) (27). RMC08L009 was obtained from the Resource for Molecular Cytogenetics, LBNL/UCSF. Briefly, 0.5 µg of pBSIISK(+)-TREX or 0.5 µg of RMC08L009 was labeled with biotin-16-dUTP (Boehringer Mannheim GmbH, Mannheim, Germany) or digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation, respectively. The mean fragment size of the nick-translated probes was between 300 bp and 600 bp. DNA probes were precipitated with 20 µg of sonicated salmon sperm DNA and 20 µg of Escherichia coli tRNA and then dissolved in 30 µl of formamide. The biotin- and digoxigenin-labeled probes were mixed at a ratio of 5/5.5 (v/v), and human Cot-1 DNA (Gibco BRL, Gaithersburg, MD) dissolved in formamide was added to the mixed solution at a concentration of 0.4 µg/µl. This mixture was heat-denatured at 75°C for 10 min and mixed with an equal volume of 4XSSC/20% dextran sulfate, and hybridized to slides of normal metaphase or prophase chromosomes at 37°C for 2 days in a humid chamber. After hybridization, the slides were washed for 15 min sequentially with 50% formamide/2XSSC at 37°C, 2XSSC, 1XSSC, and 4XSSC at room temperature, and incubated in 4XSSC/1% Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka Japan) containing avidin-FITC (15 µg/ml) and anti-digoxigenin-rhodamine (1µg/ml) (Boehringer Mannheim) at 37 °C for 40 min. Slides were washed for 10 min each in

4XSSC, 4XSSC/0.05% Triton X-100 and 4XSSC at room temperature, and for 5 min each in 2XSSC and distilled water at room temperature. Slides were then counterstained with 0.15  $\mu$ g/ml of 4,6-diamidino-2 phenylindole (DAPI) in an antifade solution.

A Nikon Eclipse E800 microscope was used for visualization of DAPI banding patterns and the hybridization signals. Digital images were acquired using a COHU high performance CCD camera (San Diego, CA) controlled with Mac Probe 3.4 software (Perceptive Scientific Instruments, Inc., Chester, UK). At least 50 metaphase or prophase cells were examined to determine the chromosomal location of TREX gene.

**Western blotting.** Proteins were separated by electrophoresis in 7.5% polyacrylamide/ SDS gels, and electrophoretically transferred to membranes for 1h. The membranes were blocked in TBS (100 mM Tris, 150mM NaCl) containing 10% nonfat dried milk and 0.1% Tween-20 for 2h. Incubation of the membranes with anti-TREX monoantibody was performed in TBS containing 5% nonfat milk and 0.1% Tween 20 for 1h and then membranes were washed with TBS containing 0.1% Tween 20 for 30 min and detected with ECL detection kit.

**DNA and RNA preparation.** All the tumor and normal tissues were obtained from Department of Otolaryngology, CPMC, Columbia University. The histopathological classification was as suggested by the WHO committee. Both normal and tumor tissues were collected at the time of surgery and snap-frozen. High molecular weight DNAs were obtained from the tissue by phenol-chloroform extraction and ethanol precipitation. Total RNAs were prepared by using TRIzol Reagent (GIBCOBRL). Sections from each of the tumors were histopathologically examined. All tumor samples contained greater than 90% tumor cells.

**Mutational analysis.** 10 PCR primers and 12 sequencing primers were designed to analyze the whole ORF of TREX. A 50  $\mu$ l reaction contained 150 ng genomic DNA, 20 pmol of each primer, 1X Expand™ High Fidelity PCR buffer (Boehringer Mannheim), and 2.6 U Expand™ High Fidelity PCR System enzyme mix (Boehringer Mannheim). After an initial denaturation for 2 min at 94 °C, 30 cycles of 20 s at 94 °C, 30 s at 60 °C, and 3 min at 68 °C, and final extension for 7 min at 68 °C were carried out in a PCR microtube thermal Cyclor (Perkin Elmer). Direct sequencing of PCR products was performed after pre-treatment by Pre-PCR sequencing kit (Amersham) using the sequencing primers as described above. All mutations were confirmed by sequencing a newly amplified product.

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### THIRD SERIES OF EXPERIMENTS

Abbreviations used herein: TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; NF- $\kappa$ B, nuclear factor- $\kappa$ B, TRAF, tumor necrosis factor receptor-associated factor; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; PBS, phosphate-buffered saline; luc, luciferase; HEK, human embryo kidney; HA, hemagglutinin; PMSF, phenylmethylsulfonyl fluoride; TRITC, trimethylrhodamineisothiocyanate; EGFP, enhanced green fluorescent protein.

EXTL3 is a member of the EXT gene family and a putative tumor suppressor gene. Here we identified the cDNA encoding mouse homolog of EXTL3 and examined the effect of its expression on nuclear factor- $\kappa$ B (NF- $\kappa$ B) activity. The mouse EXTL3 protein is 97% homologous to the human EXTL3. Northern blot analysis indicated that mouse EXTL3 is ubiquitously expressed in tissues, with highest expression in the heart, brain, and skeletal muscle. Over expression of EXTL3 enhanced tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )- and tumor necrosis factor receptor-associated factor 2 (TRAF2)-induced NF- $\kappa$ B activation. Structure-functional analysis revealed that the transmembrane region near the amino terminus was required for this effect of mouse EXTL3 on NF- $\kappa$ B activity. The results of subcellular localization studies revealed that EXTL3 was expressed predominantly at the endoplasmic reticulum. Interestingly, co-expression of EXTL3 with TRAF2 facilitates to change in distribution of EXTL3 and TRAF2 surrounded the EXTL3-containing vesicle caused by TRAF2. These results strongly suggest that EXTL3 may modulate a signal cascade mediated by TNF- $\alpha$ .

Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )<sup>3</sup> is a potent inflammatory cytokine that generates two different signals: it induces apoptosis, and it activates the transcription factor NF- $\kappa$ B (1, 2). The inhibition of NF- $\kappa$ B during TNF- $\alpha$  stimuli results in apoptosis in various cell lines which are originally resistant to TNF- $\alpha$ -induced cell death (3-5).



Therefore, activation of NF- $\kappa$ B likely induces the expression of genes that counteract apoptotic signals and prevent cell death.

Hereditary multiple exostoses syndrome (EXT) is an autosomal dominant disorder characterized by the formation of multiple cartilage-capped tumors that develop from the outgrowth plate of endochondral bone (6). Genetic linkage analysis has mapped loci for EXT at chromosomes 8q24.1 (EXT1) (7, 8), 11p11-13 (EXT2) (9, 10), and 19p (EXT3) (11). Both EXT1 (12) and EXT2 (13) genes have been identified; these proteins share extensive sequence similarity, especially at the carboxyl terminus. The three EXT-like genes, EXTL1 (14), EXTL2/EXTR2 (15, 16), and EXTL3/EXTR1 (16, 17), which also share considerable homology, have been assigned to human chromosomes 1p36.1, 1p21, 8p21, respectively. Because these chromosomal regions have been associated with high frequent loss of heterozygosity in various human cancers, it has been thought that putative tumor suppressor genes exist in these loci (18-20). Therefore, the EXT family including EXTL3 may represent a class of putative tumor suppressors.

Recently, EXT1 and EXT 2 were identified as glycosyltransferases required for biosynthesis of heparin sulfate (21, 22). However, functional role to another member of the family is still not defined. Here we report that mouse EXTL3 affects NF- $\kappa$ B activity stimulated by TNF- $\alpha$ . We also describe the subcellular localization of this protein at the endoplasmic reticulum.

#### MATERIALS AND METHODS

**Materials.** Recombinant human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was obtained from R&D Systems, Inc. (Minneapolis, MN). TRITC-conjugated concanavalin A was obtained from Sigma (St. Louis, MO). Fetal calf serum (FCS) was obtained from HyClone (Logan, UT). The NF- $\kappa$ B-dependent reporter gene construct pELAM-luc, in which the human E-selectin promoter

region (-730/+52) has been inserted into pGL3 by using SacI/BglII sites, was kindly provided by MBL (Nagoya, Japan).

5 cDNA cloning of mouse EXTL3. Mouse EXTL3 cDNA was isolated from the Mouse Brain 5'-Stretch Plus cDNA library (Clontech, California, CA) by using human EXTL3 as a probe. To extend the partial sequence, RACE was carried out as described in the manufacturer's manual (Clontech).

10 Northern blot analysis. A Northern blot filter containing mouse poly(A)+ RNAs from eight different tissues was purchased from Clontech. The filter was hybridized with the 1.2 kb EXTL3 cDNA fragment that contains the entire open reading frame as reconstructed from the RACE product.

15 Plasmid construction and transfection. To construct the expression plasmid, we PCR-amplified the full length EXTL3 cDNA fragment by using the forward primer (5'-CGCGGATCCACCATGACAGGCTATACCATGTTGCGGA-3'), which contains a BamHI site, and the reverse primer (5'-CCCAAGCTTTAGATGAAGCTTGAAGCACTTGGT-3'), which contains a HindIII site. To construct the deletion mutant lacking the N-terminal region ( $\Delta$ N), the  $\Delta$ N fragment was amplified by using the forward primer (5'-CGCGGATCCACCATGTCTCTACAAGGAGCTGATGGCCCA-3') and the reverse primer used for the full-length fragment. To construct the deletion mutant lacking the c-terminal region ( $\Delta$ C), the  $\Delta$ C fragment was amplified by using the reverse primer 5'-CCCAAGCTTGCTACCTCTTCCCGGATGGGAGCA-3' and the same forward primer as that for the full-length fragment. For the deletion mutant lacking both the N- and C-terminal portions ( $\Delta$ N&C), the  $\Delta$ N&C fragment was amplified by using the same forward primer as that for the  $\Delta$ N fragment and the reverse primer used to generate the  $\Delta$ C fragment. After digestion with BamHI and HindIII, full-length and truncated EXTL3 PCR products were ligated into pcDNA3.1(-)/Myc-His B

(invitrogen, Carlsbad, CA) such that the myc epitope tag and the 6xhis tag were in-frame for subsequent translation.

For construction of EGFP-tagged EXTL3 expression plasmids, the full-length coding region for mouse EXTL3 and the  $\Delta N$  region was PCR-amplified by using the forward primer 5'-CCCAAGCTTACCATGACAGGCTATACCATGTTGCGGA-3' and the reverse primer used for the full-length fragment described previously. In addition, the  $\Delta N$  region was generated by using the forward primer 5'-CCCAAGCTTACCATGTCTACAAGGAGCTGATGGCCCA-3' and the same reverse primer used for the full-length fragment. After digestion with *HindIII*, the full-length and  $\Delta N$  EXTL3 PCR products were ligated into pEGFP-N2 (Clontech) such that EGFP was in-frame for subsequent translation.

Full-length coding regions of mouse TRAF2 and TRAF3 were amplified by PCR and subcloned into FLAG-tagged pCR3.1 (Invitrogen). Full-length coding regions of human TRAF2 were amplified and subcloned into hemagglutinin (HA)-tagged pCDNA3 (Invitrogen).

**Cellculture and transfection.** Human embryo kidney 293 (HEK293) cells were maintained in Eagle's minimum essential medium containing 10% fetal calf serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (GIBCO-BRL, Grand Island, NY). For experiments, HEK293 cells were seeded at a density of  $10^6$  cells/dish in 10-cm culture dishes and were cultured for 3 days. Then, the cells were transfected by standard calcium phosphate co-precipitation method using commercial solution (5prime 3prime inc.).

**Preparation of nuclear extracts.** For nuclear extracts, cells were treated with or without TNF- $\alpha$  (20 ng/mL) for 1 h, washed with ice-cold PBS, and detached by using 5 mM EDTA in PBS. After pelleting, the cells were resuspended in wash buffer (10 mM Tris-HCl [pH 7.5], 130 mM NaCl, 5 mM KCl, 8 mM

MgCl<sub>2</sub>, then pelleted and resuspended in hypotonic buffer (20 mM HEPES-KOH [pH 7.9], 5 mM KCl, 0.5 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.5 mM PMSF). After incubation for 10 min on ice, the cell suspension was homogenized by using five strokes in a Dounce homogenizer. The homogenate was centrifuged for 10 min at 4000 rpm. Sedimented nuclei were resuspended in extraction buffer (20 mM HEPES-KOH [pH 7.9], 25% glycerol, 500 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5 µg/ml pepstatin A, 1.3 µg/ml spermidine) and broken by using five strokes in a Dounce homogenizer. After vortexing for 1 h, the nuclear suspension was centrifuged for 10 min at 15,000 rpm. The supernatant was dialyzed against binding buffer (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). After centrifugation, the supernatant was used as the nuclear extract.

**Electrophoretic mobility shift assays.** Electrophoretic mobility shift assays were performed by incubating an aliquot of nuclear extract containing 5 µg protein with 2 µg poly(dI-dC) (Amersham Pharmacia, Uppsala, Sweden) in assay buffer (13mM HEPES [pH 7.8], 50 mM KCl, 4.3 mM MgCl<sub>2</sub>, 10% glycerol, 0.3 mM DTT, 0.3 mM PMSF [final volume, 30 µl]). The binding reaction was started by adding endo-labeled NF-κB-specific oligonucleotide (Promega, Madison, WI) with [<sup>32</sup>P]ATP (Amersham Pharmacia) and T4 polynucleotide kinase and the reaction mixture was incubated for 30 min at room temperature. The samples were separated by polyacrylamide gel electrophoresis in low ionic-strength buffer (0.25xTris-borate-EDTA). Activated NF-κB complexes were identified by using super-shift analysis with an antibody that recognizes NF-κB subunit (Santa Cruz, California, CA).

**Luciferase assay.** For a reporter gene assay, HEK293 cells were transfected with 500 ng of the NF-κB-dependent reporter gene construct pELAM-luc, 500 ng of the internal control construct pRL-TK (Promega) and 10 µg of each expression

construct needed. DNA concentrations were kept constant by supplementation with empty vector. Cells were lysed 24 h after transfection, and reporter gene activity was determined by using the Dual luciferase assay system (Promega). Luminescence was measured in a Lumat LB 9507 (BERTHOLD GmbH & Co. KG, Bad Wildbad, Germany).

**Fluorescence microscopy.** HEK293 cells cultured on cover glasses were transfected with the EGFP-tagged EXTL3 construct and the FLAG-tagged TRAFs constructs by a standard calcium phosphate co-precipitation method. The cells were fixed with 3.7% formalin in PBS for 10 min at room temperature 24 h after transfection. The cells were washed three times with PBS and treated with 0.2% Triton X-100 in PBS for 5 min, followed by a 30 min incubation in blocking solution (PBS containing 5% BSA). After blocking, the cells were incubated with 100  $\mu$ g/mL TRITC-conjugated concanavalin A for 30 min. The cells were washed three times with PBS and then incubated with M2 anti-FLAG monoclonal antibody (Sigma) at 20  $\mu$ g/ml in 0.1% BSA in PBS for 1 h. Cells were washed three times with PBS then incubated with Cy5-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 20  $\mu$ g/ml in 0.1% BSA and 0.1% Tween 20 in PBS for 1 h. The cells were then washed with PBS and mounted on slide glasses. Fluorescence was visualized by using a Carl Zeiss LSM510 confocal laser scanning microscope (Oberkochen, Germany).

**Accession Number.** The Genbank accession number for mouse EXTL3 is AF083550.

## RESULTS

Cloning of murine EXTL3 cDNA and distribution of its mRNA in various tissues. From the mouse brain cDNA library, several colonies were selected by using human EXTL3 cDNA as a probe.

To extend the partial sequence, RACE were carried out as described in the manufacturer's manual. An open reading frame encoding a predicted protein of 918 amino acids was obtained. Mouse EXTL3 protein is 97% homologous to the human protein (Fig. 9A).

A Northern blot filter containing mouse poly(A)+ RNAs from eight different tissues was hybridized with a 1.2 kb fragment of mouse EXTL3 cDNA. A single transcript of 6.0 kb was detected in all tissues examined, with highest expression in heart, brain, and skeletal muscle (Fig. 9B). The results are consistent with those associated with human EXTL3.

**Effect of EXTL3 protein expression on NF- $\kappa$ B activity.** To investigate the effects of EXTL3 on TNF- $\alpha$ -induced NF- $\kappa$ B activation, an electrophoretic mobility shift assay was carried out. NF- $\kappa$ B activation was detected in the nuclear extract stimulated by TNF- $\alpha$  (Fig. 10A). The super shift of the band with anti-NF- $\kappa$ B p50 subunit antibody or anti-NF- $\kappa$ B p65 subunit antibody was observed. These results might indicate that the p65/p50 heterodimer was formed in TNF- $\alpha$ -treated HEK293 cells. In EXTL3-transfected cells, TNF- $\alpha$ -induced NF- $\kappa$ B activation was enhanced markedly (Fig. 10A). To confirm this finding, we also examined the effect of EXTL3 on NF- $\kappa$ B activation by using a luciferase assay. Over expression of EXTL3 enhanced TNF- $\alpha$ -induced NF- $\kappa$ B activation in a concentration-dependent manner (Fig. 10B). Similar results were obtained when EXTL3 was co-expressed with TRAF2 (Fig. 10C).

EXTL3 has a putative transmembrane region at its N-terminus and the EXT domain at its C-terminus (Fig.11A). The EXT

domain comprises two subdomains, EXT-N and EXT-C. To determine the region necessary for the enhancement of NF- $\kappa$ B activation, we constructed a series of EXTL3 deletion mutants and investigated their effect on NF- $\kappa$ B activation. The results revealed that enhancement of NF- $\kappa$ B activation was not detected in N-terminal truncated EXTL3 expressed HEK293 cells, but the C-terminal truncation mutant enhanced NF- $\kappa$ B activation (Fig. 11B and 11C). These results showed that the transmembrane region closer to the N-terminus was required for modulation of NF- $\kappa$ B activation induced by TNF- $\alpha$  or TRAF2.

**Cellular location of EXTL3 protein.** To determine the subcellular localization of EXTL3, HEK293 cells were transiently transfected with the EGFP-tagged EXTL3 expression plasmid. As shown in Fig. 11D-b, EXTL3 protein is detected at the endoplasmic reticulum. By contrast, the localization pattern of the N-terminal deletion mutant is similar to that of EGFP (Fig. 11D-a and 11D-c). These results suggested that the transmembrane region closer to the N-terminus is necessary for pre-nuclear localization.

To elucidate the role of the EXTL3 protein in TNF- $\alpha$  signaling, we examined the effects of TRAF2 and TRAF3 on the subcellular distribution of EXTL3. Although no change in EXTL3 localization was observed in HEK293 cells co-transfected with TRAF3, TRAF2 affected the subcellular distribution of EXTL3 (Fig. 12). TRAF2 caused the formation of vesicles containing EXTL3. As shown in Fig. 12H, the EXTL3 localization and the region stained with TRITC-conjugated concanavalin A clearly overlap. This result is consistent with localization of EXTL3 at the endoplasmic reticulum. However, EXTL3-containing vesicles appeared in cells co-expressing TRAF2 cells that were not stained with concanavalin A (Fig. 12D). Interestingly, TRAF2 existed at the surface of these vesicles.

## DISCUSSION

In the present study, we demonstrate that EXTL3 markedly enhances both TNF- $\alpha$ - and TRAF2-induced NF- $\kappa$ B activation, although EXTL3 slightly stimulates NF- $\kappa$ B activity in itself. The study using EXTL3 truncation mutants demonstrates that the N-terminal region containing a putative transmembrane domain is required for EXTL3-associated enhancement of NF- $\kappa$ B. Indeed, EXTL3 locates at endoplasmic reticulum, which consists with prediction based on the amino acid sequence (17). Therefore, the correct sorting of EXTL3 may be necessary for the enhancement of TNF- $\alpha$ - and TRAF2-induced NF- $\kappa$ B activation.

Previous studies demonstrated that several TRAFs associate with the TNF receptor and initiate signal transduction. TRAF2, but not TRAF3, is responsible for the activation of NF- $\kappa$ B (23). We demonstrated that EXTL3-contained vesicles appear in TRAF2 co-transfected cells but not in TRAF3 co-transfected cells. Moreover, TRAF2 exists on the surface of these vesicles. These also implicate EXTL3 in TNF- $\alpha$ -induced signal transduction. Recently, numerous protein mediating signals initiated by TNF- $\alpha$  have been identified (24). There is a possibility that EXTL3 affects the function of these proteins such as TRAF2. Several groups reported that the activation of NF- $\kappa$ B prevents apoptosis (3-5). Here, we report that EXTL3 may involved in the TNF- $\alpha$ -induced NF- $\kappa$ B activating pathway, which may help to understand the tumor suppressor activity of EXTL3.

Heparin sulfate proteoglycans are ubiquitously present on the cell surface and in the extracellular matrix. Heparin sulfate chains interact with a variety of proteins and are therefore implicated not only in various cellular responses but also in diverse physiological phenomena (25). The role of glycosaminoglycan in the transmembrane signaling induced by fibroblast growth factor is well documented (28-30). Recently, it has been reported that EXT1 and EXT 2 encode glycosyltransferases involved in the chain-elongation step



of heparin sulfate (21, 22). Therefore, another member of  
EXT family, perhaps EXTL3, also may be involved in  
glycosaminoglycan synthesis. Indeed, EXTL3 localizes to the  
endoplasmic reticulum, as EXT1 does (21, 26). Beside this,  
5 TNF- $\alpha$  has an affinity for heparin (27). These let us  
speculate that glycosaminoglycan may play a pivotal role in  
TNF- $\alpha$ -induced signal transduction as well as in fibroblast  
growth factor-induced signaling, but further studies are  
required to confirm our hypothesis.

10 The chromosomal localization of EXTL3 has been assigned to  
8p21 (16, 17, 31) and the EXTL3 gene was mapped in the  
common region of deletion in primary breast cancer (31).  
The extensive mutation search was performed using the 329  
15 primary human cancers including chondrosarcomas, breast and  
lung cancers and the results revealed that the frequent  
somatic mutation was not detected in the sporadic human  
cancers (31d), suggesting that EXTL3 may not be involved in  
tumor development and/or progression. However, loss of  
20 heterozygosity in the EXTL3 gene may cause unbalance of the  
regulation of NF- $\kappa$ B activation by TNFR-mediated signal  
transduction and eventually its loss of EXTL3 function may  
contribute to inhibition of apoptosis in primary human  
cancers. Further studies will be necessary to better  
25 understandings of association between EXTL3 function and  
tumor development and/or progression.

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